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Photometrical analysis with photosensory domains of photoreceptors in green algae

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Abstract **Chloroplast photoorientation in the green alga *Mougeotia scalaris* is controlled by blue and red light. The properties of the LOV domains of phototropin A and B were consistent with previous data of action spectra and photoreceptor lifetime for blue light-mediated photoorientation. The LOV domains of the neochromes did not bind flavin, while the domains of neochrome 2 contributed to multimer formation. The absorption spectra of the neochrome phytochrome photosensory domain with phytochromobilin were very similar to the action spectra for red light-induced photoorientation. These results indicate that phototropin and neochrome work as the blue and red photoreceptors involved in photoorientation.**

Keywords: Chloroplast photoorientation; Neochrome; Phototropin; Phytochrome; Phytochromobilin; *Mougeotia scalaris*

1. Introduction

Each cylindrical cell of the filamentous green alga *Mougeotia scalaris* contains one giant ribbon-shaped chloroplast and these have been used as material for photobiological analysis for a long time [1]. The most studied physiological response is chloroplast photoorientation. The chloroplast orientates towards incident light of weak or medium intensity. The orientation movement is mainly controlled by red light (R) irradiation [2], but blue light (B) irradiation also plays a role in the induction [3,4].

In higher plant cells with many chloroplasts, light-induced chloroplast relocation is induced mainly by B irradiation. Weak and strong light irradiation induce the accumulation and avoidance of chloroplasts, respectively [5]. It has been shown in the dicot *Arabidopsis thaliana* that the photoreceptors phototropin 1 (phot1) and 2 (phot2) are involved in the accumulation response but only phot2 is responsible for avoidance. Phototropin consists of two light sensitive, light-oxygen-voltage (LOV) domains at the N-terminus and a serine/threonine kinase domain at the C-terminus. Each LOV domain binds one flavin mononucleotide. In the fern *Adiantum capillus-veneris*, two phototropins have been identified and *Ac*phot2 has been found to mediate the avoidance response [6]. Four phototropins in the moss *Physcomitrella patens* act as photoreceptors in chloroplast relocation movement [7]. Although two phototropin genes, *MsPHOTA* and *MsPHOTB*, have so far been isolated in *M. scalaris*, their involvement in chloroplast photoorientation and their photochemical properties remain to be elucidated.

In several non-seed plants such as ferns, mosses and green algae, R- as well as B-induced chloroplast movement is known. In *A. capillus-veneris*, R-induced chloroplast relocation is controlled by phytochrome 3 (*Ac*phy3) which is a chimeric protein of a phytochrome photosensory region and a full length phototropin domain [8]. In *P. patens*, phytochrome-dependent chloroplast relocation movement is observed [9]. Four conventional phytochrome genes have been isolated, but no aberrant phytochrome-like sequences such as *Ac*phy3 had been found [10]. The R-induced chloroplast movement was reduced in *photA2photB1photB2* triple disruptants [7]. Together, these results indicate that the movement is mediated by conventional phytochromes through phototropins [7]. In *M. scalaris*, chloroplast photoorientation also shows typical R/FR photoreversibility, indicating phytochrome dependency. One conventional phytochrome (*Ms*phy1) and two neochromes (*Ms*neo1 and *Ms*neo2) which consist of a phytochrome photosensory region and phototropin-like sequences [11], have been identified. Since action spectra for chloroplast photoorientation are similar to the difference spectra of *Ms*neo1 and transient expression of both *MsNEO1* and *MsNEO2* cDNAs rescue an *Ac*phy3 mutant, it has been suggested that the photoreceptors for R-induced chloroplast photoorientation may be neochromes [11].

Recently, it was established that recombinant phytochromes with phytochromobilin (PΦB) were expressed in *E. coli* cells expressing both heme oxygenase and PΦB synthase [11,12]. Using this expression system, the recombinant phytochrome with chromophore can be analyzed photometrically. In this paper, in order to characterize the candidate photoreceptors for B- and R-mediated chloroplast photoorientation in *M. scalaris*, we performed spectral analysis of photosensory domains of several photoreceptors, two phototropins (*Ms*photA and *Ms*photB), two neochromes (*Ms*neo1 and *Ms*neo2) and one phytochrome (*Ms*phy1), using the heterologous expression system in *E. coli*.

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Abbreviations: B, blue light; CaM, calmodulin; FR, far-red light; LOV domain, light-oxygen-voltage domain; Neo1-PL, photosensory domain of neochrome 1; Neo2-PL, photosensory domain of neochrome 2; PhotA-L, LOV domains of phototropin A; PhotB-L, LOV domains of phototropin B; Phy1-P, phytochrome photosensory domain of phytochrome 1; PΦB, phytochromobilin; R, red light

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2. Materials and methods

2.1. Genes

cDNAs of *PHOTA* (Accession Number AB206963), *PHOTB* (AB206964), *NEO1* (AB206961), *NEO2* (AB206962) and *PHY1* (AB206965) of *Mougeotia scalaris* were used.

2.2. Recombinant polypeptide purification and spectroanalysis

For expression of the recombinant polypeptides of PhotA-L (49–399), PhotB-L (36–373), Neo1-PL (1–1067), Neo2-PL (1–1056) and Phy1-P (1–580), the cDNAs were amplified by PCR in such as way as to add restriction enzyme sites at both ends that enabled easy introduction into pCAL-n-EK with six his-tag sequences. After construc-

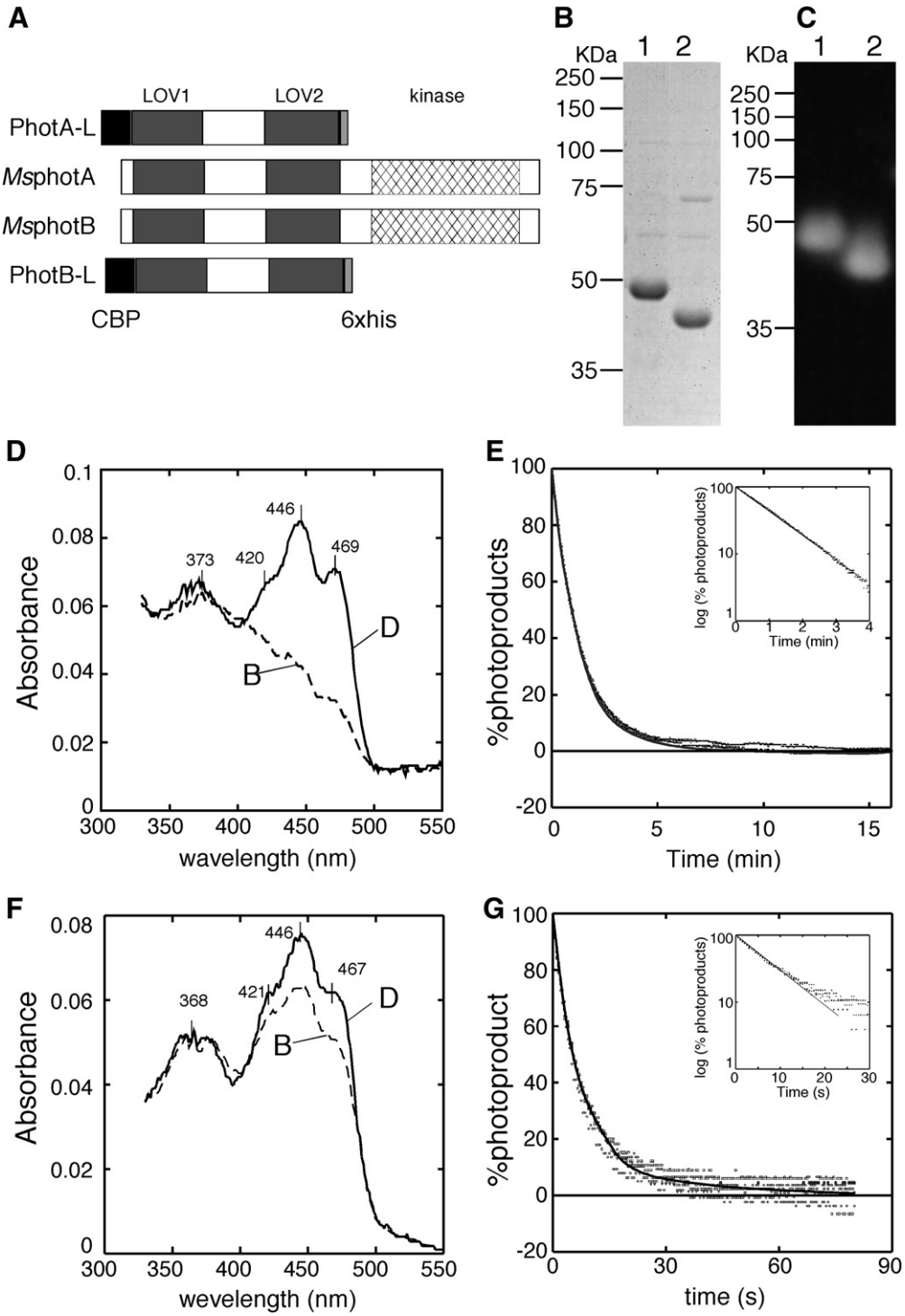

Fig. 1. Spectral analysis of LOV domains in *Ms*photA and *Ms*photB. (A) Schematic drawing of *Ms*photA, *Ms*photB and recombinant polypeptides (PhotA-L, PhotB-L). (B, C) CBB staining (B) and immunoblotting with anti-his antibody (C) of purified recombinant polypeptides of PhotA-L (lane 1) and PhotB-L (lane 2). (D) Absorption spectra of PhotA-L before (D) and after (B) B irradiation. (E) Time course of % photoproducts of PhotA-L after the B irradiation was turned off. Inset, single exponential plot in panel E. Absorptions at 450 nm were measured after the irradiation with saturated B. (F) Absorption spectrum of PhotB-L before (D) and after (B) B irradiation. (G) Time course of % photoproducts of PhotB-L after the B irradiation was turned off. Inset, single exponential plot in panel G. Other details are the same as (E).

tion each vector was co-transformed together with a PΦB-generating vector of pCDF with *Ac*HO1 and *Ac*HY2 into Rosetta™ *E. coli* cells (Merck, Darmstadt, Germany). Recombinant protein of *Ms*photA, *Ms*photB, *Ms*neo2 and *Ms*phy1 induced by isopropyl-1-thio-β-D-galactopyranoside was purified using a calmodulin (CaM) resin column (Stratagene, CA, USA). In the case of *Ms*neo1, the recombinant protein was purified using a combination of Profinity™ IMAC Ni-charged Resin (BioRad, CA, USA) and CaM resin columns. Photometrical analysis was performed using a photometer (Biospec 1650, Shimazu, Japan) or a diode array spectrophotometer (DU7500, Beckman Coulter, CA). Red, far-red, and blue illumination was obtained using light emitting diodes of red (660 nm; SLP-838A-37, Sanyo Semiconductor Corp., Japan), far-red (771 nm; HE7601SG, Hitachi, Japan), and blue (475 nm; E1L53-3B, Toyota-gosei, Japan) wavelengths.

2.3. Gel chromatography

Gel chromatography was performed using a Superose 6 10/300 GL column of a chromatography system (ÄKTAexplorer 10S, GE Healthcare, NJ, USA). The column was equilibrated with elution buffer (20 mM Na-PO_4 buffer pH 7.4, 500 mM NaCl). At a flow rate of 0.3 ml min^{-1}, the column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), albumin (67 kDa) and chymotrypsinogen A (25 kDa). Protein elution was monitored by absorbance at 280 and 660 nm simultaneously.

3. Results

3.1. Spectroanalysis of phototropin LOV domains

Initially, we carried out photometrical analysis of the LOV domains of *Ms*photA and *Ms*photB (PhotA-L, PhotB-L; Fig. 1A). Recombinant LOV domains were purified to near homogeneity, verified by CBB staining (Fig. 1B) and by immunoblotting (Fig. 1C). Absorption spectra of both the LOV domains showed typical flavin absorption spectra (Fig. 1D, F). By B-irradiation, the polypeptides were bleached and soon

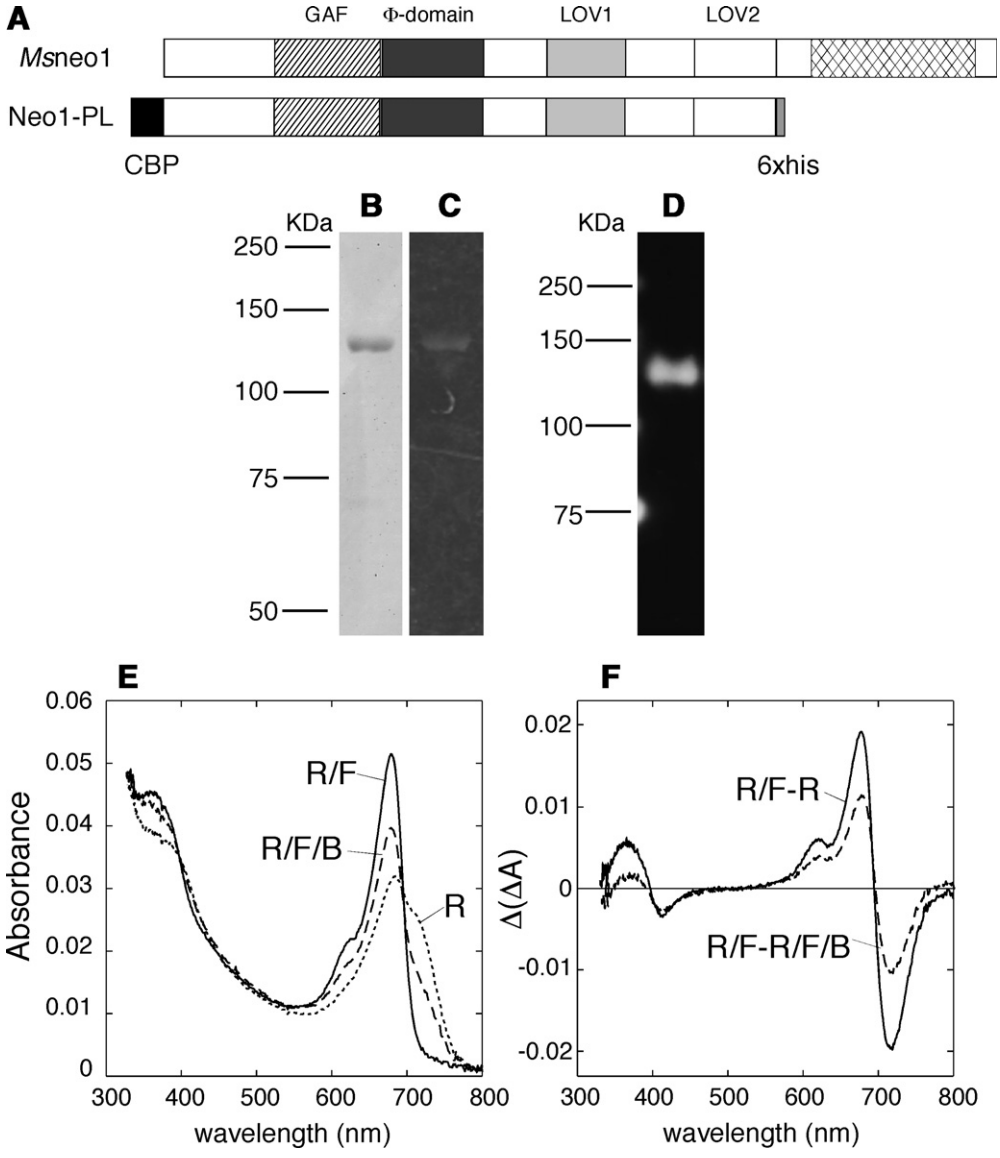

Fig. 2. Spectral analysis of recombinant photosensory region of *Ms*neo1. (A) Schematic drawing of *Ms*neo1 and recombinant polypeptides. The Neo1-PL has a phytochrome sensory domain and a phototropin LOV domain-like region. (B–D) CBB staining (B) of SDS–PAGE, Zn staining (C) and immunoblotting with anti-poly-histidine antibody (D). (B) and (C) were the same gel. (E) Absorption spectra of Neo1-PL with PΦB. The sample was sequentially irradiated with red (R), far-red (F), and blue (B) light. The measured sample was the same fraction of (B–D). (F) Difference spectra of Neo1-PL. The spectra were obtained from subtraction of R/F minus R (R/F − R) and R/F minus R/F/B (R/F − R/F/B) in (E).

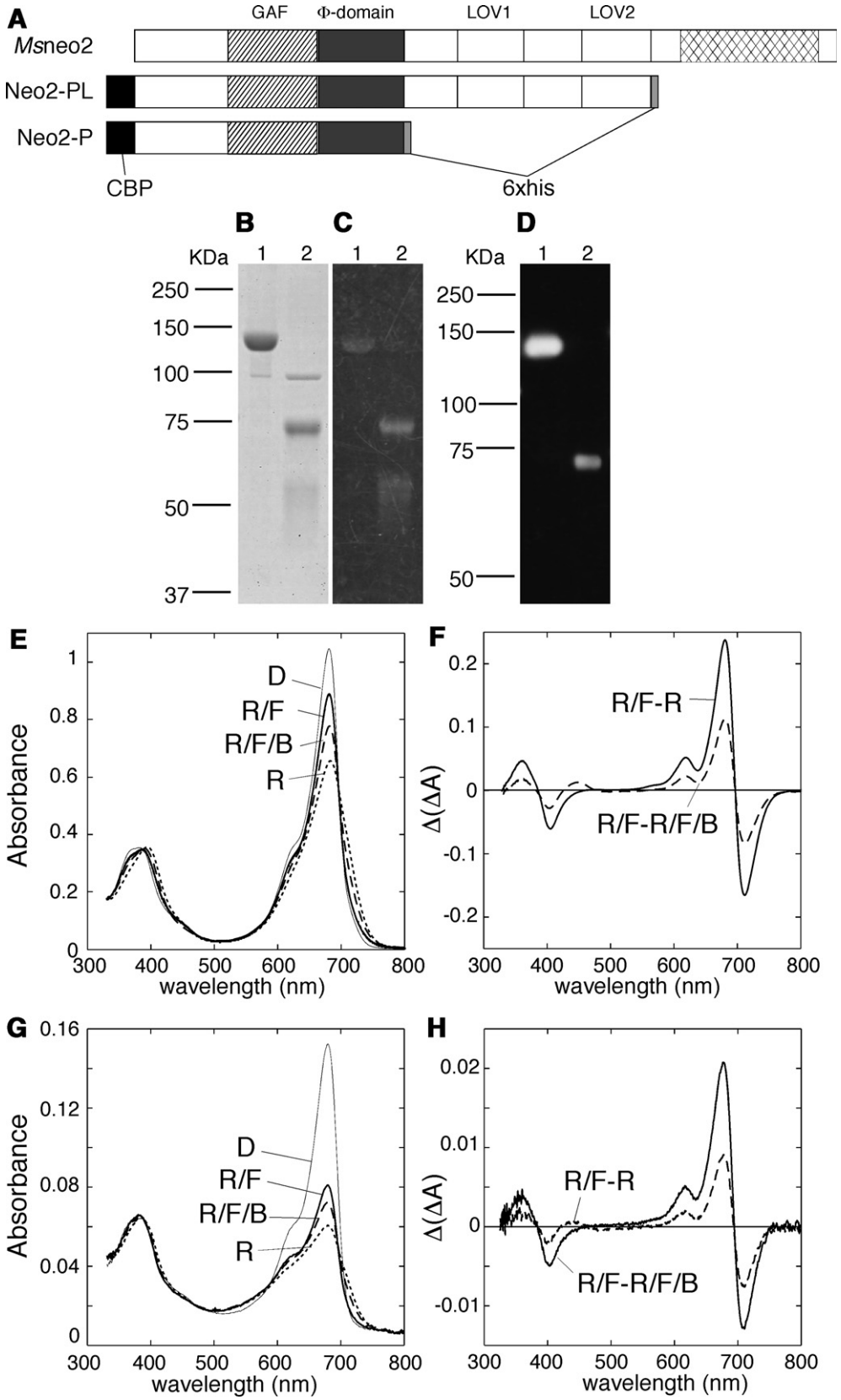

Fig. 3. Spectral analysis of recombinant photosensory region of *Ms*neo2. (A) Schematic drawing of *Ms*neo2 and recombinant polypeptides. Neo2-PL has a phytochrome sensory domain and a phototropin LOV domain-like region and the Neo2-P has a phytochrome sensory domain. (B–D) CBB staining (B) of SDS–PAGE, Zn staining (C) and immunoblotting with anti-poly-histidine antibody (D) of Neo2-PL (lane 1) and Neo2-P (lane 2). (B) and (C) were the same gel. (E) Absorption spectra of Neo2-PL with PΦB. The sample was measured before light irradiation (D). Other details are the same as in Fig. 2E. (F) Difference spectra of Neo2-PL. Other details are the same as Fig. 2F. (G) Absorption spectra of Neo2-P with PΦB. Other details are the same as in E. (H) Difference spectra of Neo2-P. Other details are the same as in Fig. 2F.

returned to a dark-adapted spectrum. In the case of PhotA-L, 50% and 90% of the bleached polypeptides returned to the dark state within 50 and 165 s, respectively (Fig. 1E). In PhotB-L, 50% and 90% of the activated LOV domain reverted within 5.5 and 20 s, respectively (Fig. 1G).

3.2. Spectroanalysis of neochrome photosensory domains

Neochromes may be photoreceptors for R-induced orientation movement. But, they may also be one of the candidates for a B-receptor in chloroplast photoorientation since they include phototropin-like sequences. We performed spectral analysis of *Ms*neo1 (Fig. 2). Truncated *Ms*neo1 (Neo1-PL), which consists of a phytochrome photosensory region and a LOV domain-like region, were expressed in PΦB-generating *E. coli* (Fig. 2B, D). The polypeptides treated with Zn solution after SDS–PAGE showed fluorescence under ultraviolet illumination, suggesting that Neo1-PL attached to PΦB covalently (Fig. 2C). Neo1-PL, which showed R/FR photoreversibility (data not shown), had a maximum peak at 678 nm in the dark or after far-red light (FR) irradiation, and showed a shoulder around the FR region after R or B irradiation (Fig. 2E). Difference spectra of FR minus R and FR minus B showed a maximum peak at 677 nm and a minimum peak at 716 nm (Fig. 2F). Although Neo1-PL has the phototropin LOV domain-like region, we did not detect any flavin-contributing spectra in the violet to B wavelengths. When expressed Neo1-PL was incubated with excess amount of FMN, we could not detect Neo1-PL with FMN (data not shown), suggesting that LOV domain of Neochrome 1 can not bind FMN although the LOV1 domain has the conserved cysteine [13].

Next, we performed similar analysis with *Ms*neo2 (Fig. 3). Neo2-PL was also affinity-purified to near homogeneity (Fig. 3B–D, lane 1), but the Neo2-P fraction included some degraded protein (Fig. 3B–D, lane 2). Neo2-PL and Neo2-P bound with PΦB covalently (Fig. 3C) and showed R/FR photoreversibility (data not shown). Dark-adapted Neo2-PL had a maximum peak at 680 nm, but the spectra after R- and B-illumination showed bleaching without a change in peak wavelength (Fig. 3E), whereas difference spectra were obtained (Fig. 3F). The maximum peak of difference spectra was at 680 nm and the minimum at 711 nm. The absorption of the violet-B region did not show typical flavin spectra. Even when the polypeptides were irradiated with B, the difference spectrum did not show typical photobleaching as usually seen with a flavin *cis*-adduct around the violet-B region though a small peak appeared at around 400–450 nm. In the difference spectrum of F minus B of Neo2-P a small peak was observed at around 400–450 nm like Neo2-PL (Fig. 3G, H), indicating that the small peak does not originate from LOV domain-like sequences.

A size fractionation profile of the Neo2-PL on a calibrated Superose 6 10/300 GL column showed one peak of 660 nm with a maximum at 14.1 ml identical to the peak of 280 nm

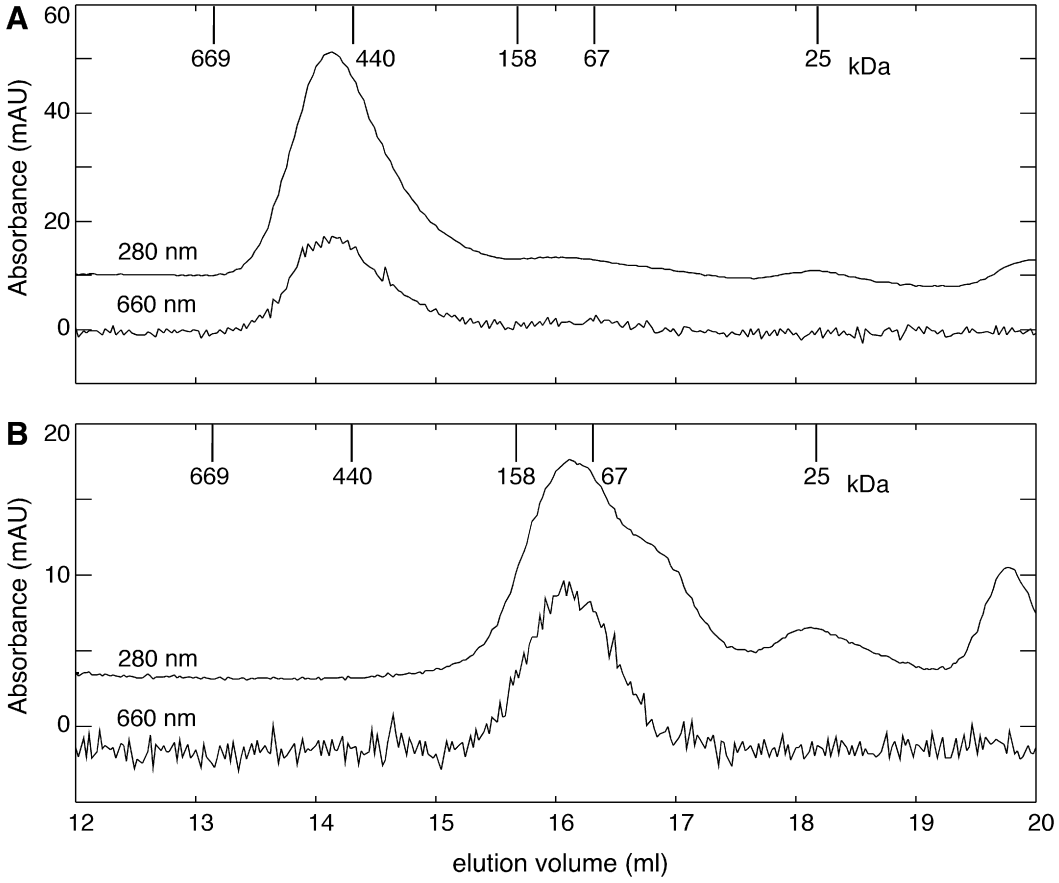

Fig. 4. Size fractionation of Neo2-PL and Neo2-P. Elution profiles of Neo2-PL (A) and Neo2-P (B) on Superose 6 10/300 GL column were obtained. The flow rate was 0.3 ml min^{-1} and the elution buffer was 20 mM Na-PO_4 pH 7.4 and 500 mM NaCl.

(Fig. 4A); this corresponds to an apparent size of about 500 kDa. Since the monomer form of the Neo2-PL is approximately 120 kDa (see Fig. 3B lane 1), the Neo2-PL is existing as a tetramer in the solution. In contrast, the elution peak of the Neo2-P (about 70 kDa) was taken at 16.1 ml corresponding to a size of 80 kDa (Fig. 4B), indicating that the Neo2-P exists as a monomer in solution. These results imply that the LOV domain-like regions of neochrome 2 act as multimerization sites.

3.3. Spectroanalysis of phytochrome photosensory domain

Finally, in order to compare conventional phytochrome spectra, we performed spectral analysis of the photosensory region of *Ms*phy1 with PΦB (Fig. 5). The purified polypeptides (Fig. 5B, D) were bound covalently with PΦB. Pr and Pfr forms have maximum peaks at 656 nm and 720 nm, respectively (Fig. 5E). The difference spectrum has a maximum peak at 652 nm and a minimum peak at 724 nm (Fig. 5F), and the difference wavelength is 72 nm. When Phy1-P was irradiated with B, about 35% converted to Pfr compared to induction with saturating R-irradiation but the contribution that flavin made to the spectrum was not measured (Fig. 5F).

4. Discussion

The action spectrum of B-mediated chloroplast photoorientation is very similar to that of flavin [1]. Although *Ms*neo1 and *Ms*neo2 have LOV domain-like sequences, B-perception by flavin in Neo1-PL and Neo2-PL was not observed, consistent with a previous report in which neither the LOV1 or LOV2 domains from either *Ms*neo1 and *Ms*neo2 showed flavin-binding [11]. B induction should be usually mediated by phototropins. Both *Ms*photA and *Ms*photB showed flavin-like spectra and their dark reversions were consistent with previous photobiological data of photoreceptor lifetime for B-induced

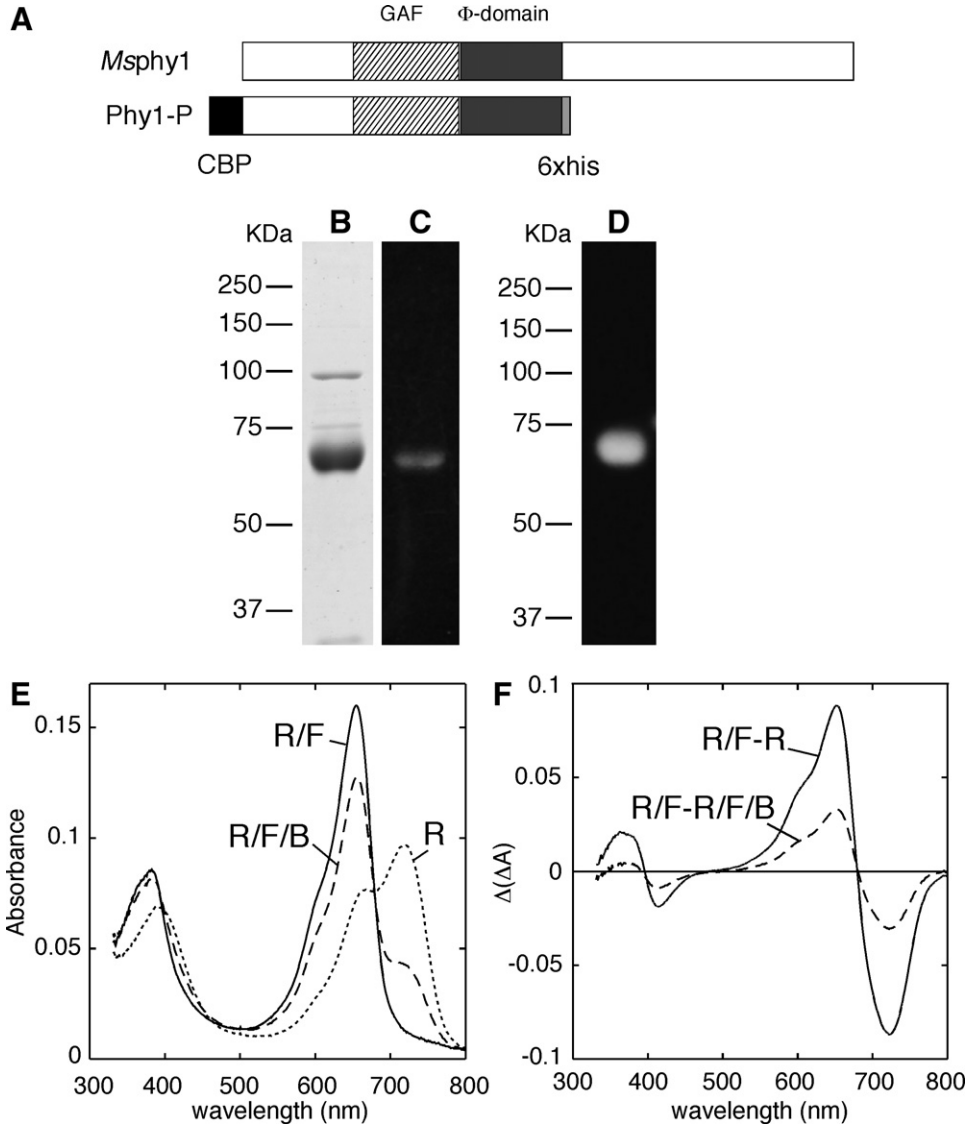

Fig. 5. Spectral analysis of phytochrome sensory region of *Ms*phy1. (A) Schematic drawing of *Ms*phy1 and recombinant polypeptide (Phy1-P). Recombinant polypeptide of Phy1-P has a phytochrome photosensory domain. (B–D) CBB staining (B) of SDS–PAGE, Zn staining (C) and immunoblotting with anti-poly-histidine antibody (D). (B) and (C) were the same gel. (E) Absorption spectra of Phy1-P with PΦB. Other details are the same as in Fig. 2E. (F) Difference spectra of the Phy1-P. Other details are the same as in Fig. 2F.

chloroplast photoorientation [3]. Since dark recovery of photA-L was longer than that of photB-L, they might have different physiological function like phot1 and phot2 in *A. thaliana* [14,15].

In contrast, the most effective wavelength in R-mediated chloroplast photoorientation is at 679 nm [2]. Recombinant proteins of *Ms*neo2 and *Ms*phy1 have been demonstrated photometrically to measure alongside phycocyanobilin but not with PΦB [11,16]. In this report, we demonstrate for the first time that recombinant *Ms*phy1, *Ms*neo1 and *Ms*neo2 with PΦB can be obtained and analyzed photometrically. The results show that the maximum peak of Pr of *Ms*phy1, *Ms*neo1 and *Ms*neo2 with PΦB were 656, 678 and 680 nm, respectively. Furthermore, *Ms*neo1 as well as *Ms*neo2 worked as a R-receptor when their cDNA were expressed transiently in a *rap* mutant which was deficient in the *Ac*phy3 gene [11]. Although conventional phytochromes should be the photoreceptors for chloroplast relocation in moss [7], *Ms*phy1 might be not the R-photoreceptor since the maximum peak of recombinant *Ms*phy1 with PΦB and phycocyanobilin was 656 and 646 nm [16] respectively, too short compared with the peak of the action spectra.

The LOV1 domain of phot2 in *A. thaliana* works as an attenuator for kinase activity [17] and that of oat, *As*phot1, acts as a dimerization site [18]. The LOV domains of both neochromes do not work as photoreceptive domains, while the LOV domain of *Ac*phy3 can absorb B [19]. However, the LOV domain of *Ms*neo2 is responsible for multimer formation, although it does not bind any flavin and has the conserved cysteine.

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References

[1] Haupt, W. and Häder, D.-P. (1994) Photomovement in: Photomorphogenesis in Plants (Kendrick, R.E. and Kronenberg, G.H.M., Eds.), second ed, pp. 707–732, Kluwer Academic Publishers, Netherlands.
[2] Haupt, W. (1959) Die Chloroplastendrehung bei *Mougeotia* I. Mitteilung Über den quantitative und qualitativen Lichtbedarf der Schwachlichtbewegung. Planta 53, 484–501.
[3] Gabryś, H., Walczak, T. and Haupt, W. (1984) Blue-light-induced chloroplast orientation in *Mougeotia*. Evidence for a separate sensor pigment besides phytochrome. Planta 160, 21–24.
[4] Gabryś, H., Walczak, T. and Haupt, W. (1985) Interaction between phytochrome and the blue light photoreceptor system in *Mougeotia*. Photochem. Photobiol. 42, 731–734.
[5] Wada, M., Kagawa, T. and Sato, Y. (2003) Chloroplast movement. Annu. Rev. Plant Physiol. Plant Mol. Biol. 54, 455–468.
[6] Kagawa, T., Kasahara, M., Abe, T., Yoshida, S. and Wada, M. (2004) Function analysis of phototropin2 using fern mutants deficient in blue light-induced chloroplast avoidance movement. Plant Cell Physiol. 45, 416–426.
[7] Kasahara, M., Kagawa, T., Sato, Y., Kiyosue, T. and Wada, M. (2004) Phototropins mediate blue and red light-induced chloroplast movements in *Physcomitrella patens*. Plant Physiol. 135, 1388–1397.
[8] Kawai, H., Kanegae, T., Christensen, S., Kiyosue, T., Sato, Y., Imaizumi, T., Kadota, A. and Wada, M. (2003) Responses of ferns to red light are mediated by an unconventional photoreceptor. Nature 421, 287–290.
[9] Kadota, A., Sato, Y. and Wada, M. (2000) Intracellular chloroplast photorelocation in the moss *Physcomitrella patens* is mediated by phytochrome as well as by a blue-light receptor. Planta 210, 932–937.
[10] Mittmann, F., Brücker, G., Zeidler, M., Repp, A., Abts, T., Hartmann, E. and Hughes, J. (2004) Targeted knockout in *Physcomitrella* reveals direct actions of phytochrome in the cytoplasm. Proc. Natl. Acad. Sci. USA 101, 13939–13944.
[11] Suetsugu, N., Mittmann, F., Wagner, G., Hughes, J. and Wada, M. (2005) A chimeric photoreceptor gene, NEOCHROME, has arisen twice during plant evolution. Proc. Natl. Acad. Sci. USA 102, 13705–13709.
[12] Mukougawa, K., Kanamoto, H., Kobayashi, T., Yokota, A. and Kohchi, T. (2006) Metabolic engineering to produce phytochromes with phytochromobilin, phycocyanobilin, or phycoerythrobilin chromophore in *Escherichia coli*. FEBS Lett. 580, 1333–1338.
[13] Salomon, M., Christie, J.M., Knieb, E., Lempert, U. and Briggs, W.R. (2000) Photochemical and mutational analysis of the FMN-binding domains of the plant blue light receptor, phototropin. Biochemistry 39, 9401–9410.
[14] Kasahara, M., Swartz, T.E., Olney, M.A., Onodera, A., Mochizuki, N., Fukuzawa, H., Asamizu, E., Tabata, S., Kanegae, H., Takano, M., Christie, J.M., Nagatani, A. and Briggs, W.R. (2002) Photochemical properties of the flavin mononucleotide-binding domains of the phototropins from Arabidopsis, rice, and *Chlamydomonas reinhardtii*. Plant Physiol. 129, 762–773.
[15] Sakai, T., Kagawa, T., Kasahara, M., Swartz, T.E., Christie, J.M., Briggs, W.R., Wada, M. and Okada, K. (2001) Arabidopsis nph1 and npl1: Blue light receptors that mediate both phototropism and chloroplast relocation. Proc. Natl. Acad. Sci. USA 98, 6969–6974.
[16] Jorissen, H.J., Braslavsky, S.E., Wagner, G. and Gärtner, W. (2002) Heterologous expression and characterization of recombinant phytochrome from the green alga *Mougeotia scalaris*. Photochem. Photobiol. 76, 457–461.
[17] Matsuoka, D. and Tokutomi, S. (2005) Blue light-regulated molecular switch of Ser/Thr kinase in phototropin. Proc. Natl. Acad. Sci. USA 102, 13337–13342.
[18] Salomon, M., Lempert, U. and Rüdiger, W. (2004) Dimerization of the plant photoreceptor phototropin is probably mediated by the LOV1 domain. FEBS Lett. 572, 8–10.
[19] Christie, J.M., Salomon, M., Nozue, K., Wada, M. and Briggs, W.R. (1999) LOV (light, oxygen, or voltage) domains of the blue-light photoreceptor phototropin (nph1): binding sites for the chromophore flavin mononucleotide. Proc. Natl. Acad. Sci. USA 96, 8779–8783.